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THE EFFECTS OF 2560 r OF X-RAYS ON SPERMATOGENESIS IN THE MOUSE [1]

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The effects of irradiation on the mammalian testis have been the subject of numerous investigations (for example Regaud and Blanc, 1906; Hertwig, 1938; Eschenbrenner and Miller, 1950; Oakberg, 1955; and Bryan and Gowen, 1956). The more recent papers of this series have been concerned with quantitative aspects of the problem. The foregoing studies have established the fact that the spermatogonia are the most radiation-sensitive constituents of the seminiferous tubules. After exposure to radiation, spermatogonial proliferation is progressively reduced and the frequency of spermatogonia declines to a very low level. Following this irradiation-induced decrease in spermatogonia, the other cell types (spermatocytes, spermatids and sperm) disappear in the order of their development. Knowledge of the nature of the spermatogonial response is therefore of considerable importance to an approach toward an understanding of the action of irradiation on cells and tissues. Evidence has accrued which suggests that spermatogonial necrosis may be an important factor (Regaud and Lacassagne, 1927; Hertwig, 1938; and Oakberg, 1955). In this regard conclusions based on tracer studies must also be considered. The studies of Holmes (1947), Howard and Pelc (1953), Forssberg and Klein (1954), Smellie *et al.* (1955) and others, clearly show that irradiation effectively inhibits DNA synthesis together with mitotic activity. Furthermore the data of Howard and Pelc (1953) indicate that the mitotic inhibition (or delay) is brought about by the failure of cells in interphase to enter the synthetic phase, rather than by interruption of synthetic processes already going on. These studies would suggest that in the case of the testis, the cessation of spermatogonial activity (through inhibition of DNA synthesis) should be a major factor in bringing about the irradiation-induced depletion of spermatogonia. The data of Bryan and Gowen (1956), derived from quantitative histological and from cytophotometric studies, are in accord with these ideas—as are the earlier conclusions of Eschenbrenner and Miller (1950) and Shaver (1953). There are, then, two rather different responses to irradiation which have been advanced as explanations for the observed behavior of irradiated mammalian seminiferous tubules. The important

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point is: what are the relative levels of importance which may be ascribed to either process? It appeared likely that a comparison of results following exposure to different dose levels of x-rays would shed more light on the nature of any relationship between these proposed mechanisms. In our previous paper results obtained following exposure to 320 r of x-rays were reported. This present paper reports data obtained following exposure to a high dose of x-rays (2560 r). These data are, where feasible, presented together with corresponding data from our 320 r experiment. As will be seen, the available evidence suggests that both mechanisms play a role in the observed radiation response of spermatogonia, the level of importance ascribable to either one depending upon the dosage levels of radiation employed.

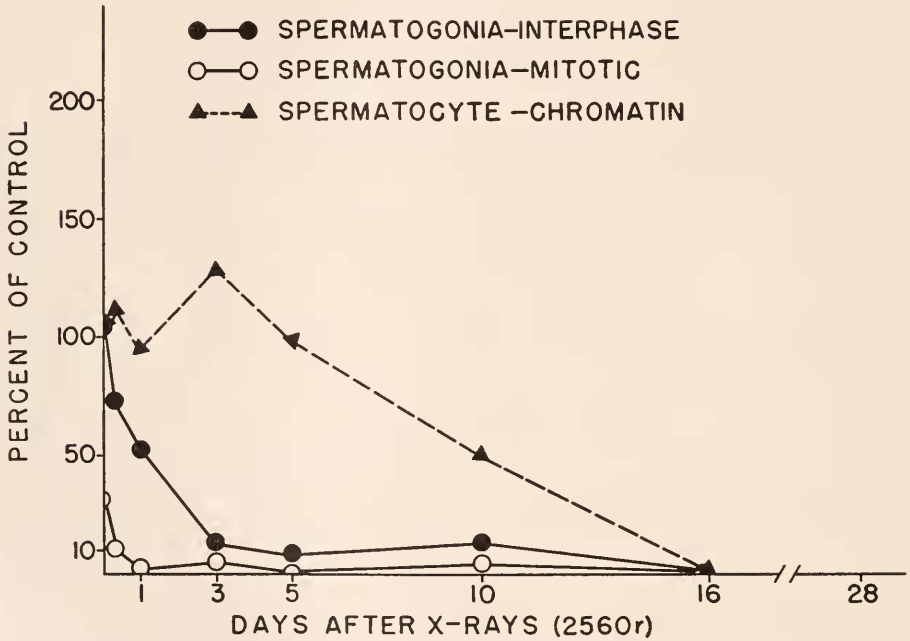

FIGURE 1. Strain Ba. Incidence of spermatogonia and spermatocytes at different times following 2560 r of x-rays.

MATERIALS AND METHODS

The animals chosen were 58-day-old males of strains BALB/Gw (hereinafter referred to as Ba) and S. These inbred strains of mice differ in their sensitivity to mouse typhoid. The experimental animals were irradiated in plastic tubes and were exposed to a dose of 2560 r (250 pkv, 30 ma; filtration 0.25 mm. Cu, 1 mm. Al; anode-target distance 47.5 cm., dose rate 430 r/min.). The irradiation was delivered to the pelvic region only, the rest of the body being shielded with lead. These conditions of irradiation are, except for the x-ray dose, identical with those of our previous studies (Bryan and Gowen, 1956).

Control and irradiated animals were killed at 1, 8 and 24 hours, 3, 5, 10, 16

and 28 days following exposure. The 28-day material is missing from the S series due to death of animals prior to this sampling time. From each animal the testes were rapidly removed and weighed. One testis was then fixed in Carnoy's acetic-alcohol (1:3) and the other used for dry weight determinations.

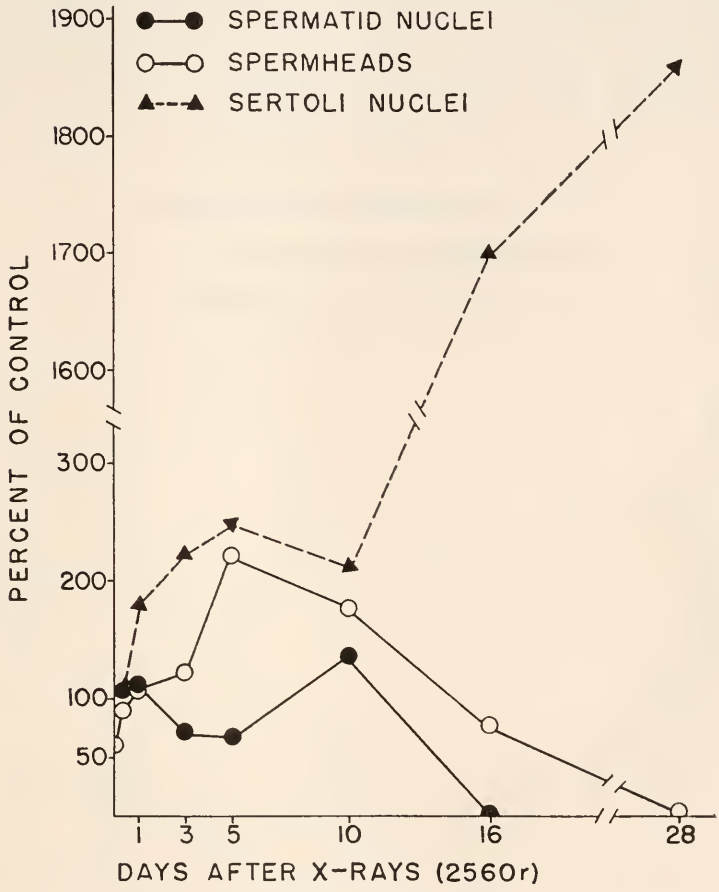

FIGURE 2. Strain Ba. Incidence of spermatids, sperm and Sertoli nuclei at different times following 2560 r of x-rays.

The histological material was processed, and slides were stained, as described earlier (Bryan and Gowen, 1956). As in our previous work, the procedure of Chalkley (1943) was used to obtain estimates of the relative areas of the tubules occupied by each stage of spermatogenesis. This procedure was also used to provide data with respect to spermatogonial and non-spermatogonial necrosis during the first 24 hours following exposure to x-rays.

RESULTS

The data obtained are summarized in Tables I–III. Data pertaining to both strains are presented together for ease of comparison. In Table I all values are

expressed in terms of per cent of control values. These data are also expressed in graphical form in Figures 1–4.

The data of Table I indicate that the relative area occupied by spermatogonia in interphase undergoes little change during the first hour after irradiation. Thereafter there is a pronounced and progressive decline which reaches a low point by 5 days following exposure. The data further suggest that there may be an abortive attempt at regeneration during the period of 5–10 days after x-rays, followed by a further decline (absence of spermatogonia at 16 days). With respect to the mitotically active spermatogonia, a marked contrast in response is evident.

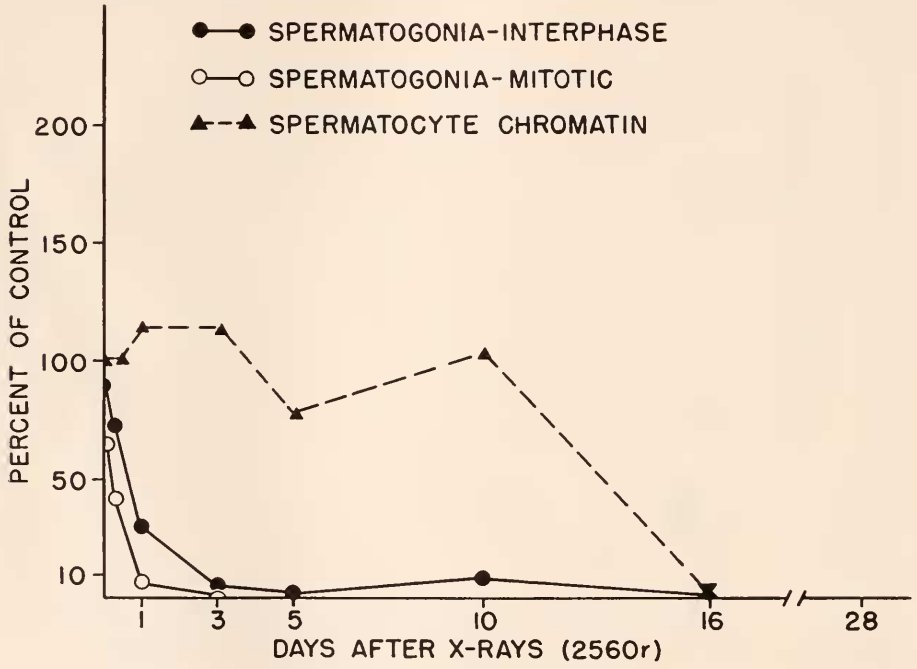

FIGURE 3. Strain S. Incidence of spermatogonia and spermatocytes at different times following 2560 r of x-rays.

Thus by one hour after irradiation the area occupied by this class has declined to 65% of the control in the case of strain S and 30% of the control in strain Ba. In both strains at one day after exposure, the area has further declined to less than 10% of the control value. Thereafter no spermatogonial mitotic activity was recorded for strain S during the remainder of the experiment. In the case of strain Ba, the response is essentially the same except that a low level of spermatogonial activity (4% of control) was encountered in the 10-day material.

Two other cell classes disappear from the seminiferous epithelium following exposure to 2560 r of x-rays. These are the spermatocytes and spermatids. The respective areas occupied by these cells have declined to zero levels by 16 days after exposure.

In view of the recorded differences in behavior of the sperm fraction of the two strains (see Table I, and Figures 2 and 4), it is unfortunate that 28-day material from strain S was not available. The response over the period of 1–10 days is rather similar. Over the period 10–28 days, strain Ba data indicate a progressive decline to a very low level at 28 days whereas strain S, in contrast, shows a marked increase during the 10–16-day period.

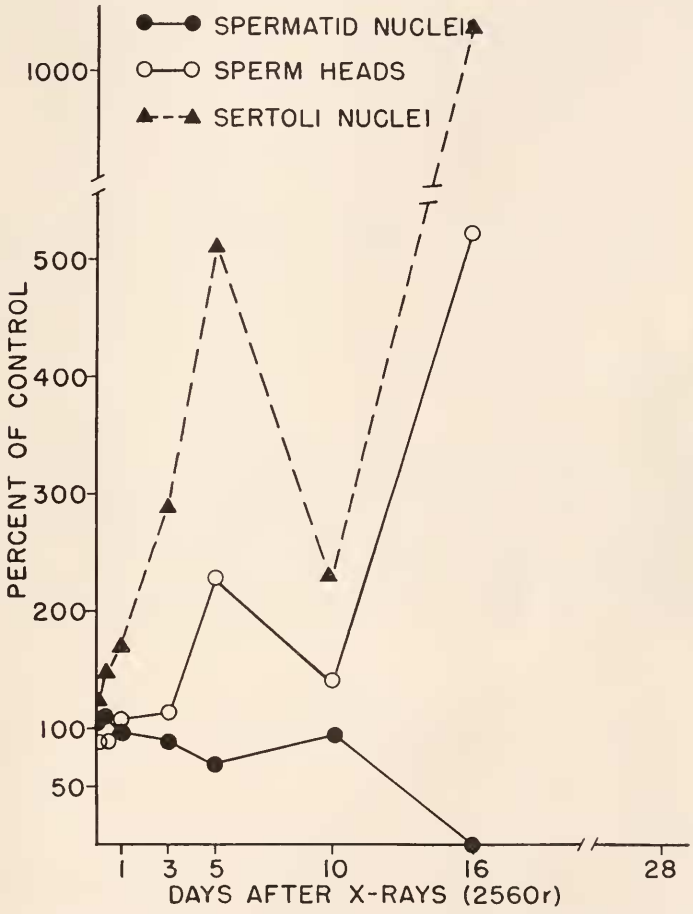

FIGURE 4. Strain S. Incidence of spermatids, sperm and Sertoli nuclei at different times following 2560 r of x-rays.

The data pertaining to the Sertoli cell fraction indicate that the trend is similar in both strains but differs in magnitude. Thus in the case of strain Ba the relative area occupied by this class undergoes a steady increase over the period 0–5 days at which time the level reached is about 2.5 times the control. This value then falls to about 2 times the control value by 10 days, and then increases again reaching at 16 days a level about 17 times the control value. In the case of strain S

TABLE I

Frequency of cell types at various times after 2560 r of x-rays[2]

Stage and strain		1 hour	8 hours	1 day	3 days	5 days	10 days	16 days	28 days
Spermatogonia in interphase	Ba	103.5	72.5	51.6	13.4	7.7	13.3	0.0	0.0
	S	90.1	73.5	29.9	5.5	1.1	8.9	0.0	—
Spermatogonia in mitosis	Ba	30.5	9.7	1.7	5.1	0.0	4.3	0.0	0.0
	S	65.4	41.9	6.9	0.0	0.0	0.0	0.0	—
Spermatocyte chromatin	Ba	105.6	111.4	95.1	128.5	99.2	49.7	0.0	0.0
	S	100.9	100.0	115.1	114.8	79.0	104.0	0.0	—
Spermatid nuclei	Ba	113.5	105.6	110.7	72.4	68.1	138.1	0.0	0.0
	S	106.2	110.2	97.8	91.6	69.7	97.7	0.0	—
Sperm heads	Ba	59.9	88.9	106.3	120.8	222.2	178.2	78.4	4.4
	S	89.8	91.2	107.3	113.5	227.5	139.7	521.6	—
Sertoli nuclei	Ba	97.9	107.6	181.6	223.2	250.1	212.5	1,702	1,860
	S	121.6	143.6	172.1	293.4	510.5	227.2	1,036	—

[2] Values expressed as per cent of controls.

the corresponding values are at 5 days 5 times, and at 10 days 2.2 times the control levels. The value attained by 16 days is about 11.4 times the control level. The cause of this striking difference between the strains, at 5 days, is not clear. These changes in relative areas of the Sertoli fraction are, within limits, reflections of changes in area of the spermatogenic cells.

In Table II are presented the data with respect to spermatogonial and non-spermatogonial necrosis. Data from our previous experiment (320 r) are included

TABLE II

Spermatogonial and non-spermatogonial necrosis at various times after 320 r or 2560 r of x-rays

Strain and time after x-rays		320 r		2560 r	
		% spermatogonial necrosis	% non-spermatogonial necrosis	% spermatogonial necrosis	% non-spermatogonial necrosis
Control	Ba	3.8	2.1	3.8	2.1
	S	4.0	2.5	4.0	2.5
1 hour	Ba	3.4	3.0	6.0	3.7
	S	5.8	2.4	6.5	1.2
8 hours	Ba	9.7	2.2	15.3	3.0
	S	8.8	1.7	14.9	2.0
24 hours	Ba	7.4	2.3	0.0	4.5
	S	4.6	2.2	0.0	3.2

in this table for purposes of comparison. All data in this table are expressed as percentages.

Changes in area occupied by the spermatogonial fraction (normal interphasic + mitotic + necrotic spermatogonia) following exposure to x-rays are presented in summary form in Table III.

TABLE III

A comparison of expected and observed frequencies of spermatogonia at various times following exposure to x-rays of different dose levels

Time after x-rays	Strain					
	Ba			S		
	% total spermatogonia	Difference from control	Difference as % of control	% total spermatogonia	Difference from control	Difference as % of control
Control	12.35			15.10		
320 r 1 hour	14.47	+2.12	17.17	13.55	−1.55	10.26
8 hours	9.89	−2.46	19.92	12.29	−2.81	18.61
24 hours	10.39	−1.96	15.87	6.78	−8.32	55.10
2560 r 1 hour	9.11	−3.24	26.23	13.21	−1.79	11.85
8 hours	10.27	−2.08	16.84	11.61	−3.49	23.11
24 hours	3.32	−9.03	73.12	3.01	−12.09	80.07

DISCUSSION

In the case of the testis it is clear that any treatment which interferes with, or prevents, spermatogonial mitotic activity will bring about partial or complete maturation depletion of the seminiferous tubules. Temporary depletion will follow if, for a short period of time, the level of spermatogonia is reduced much below normal thereby preventing the quantitative replacement of the spermatocyte fraction. Such a reduction may be brought about either by inhibition of chromosomal reduplication (and therefore of mitosis), a relatively high level of spermatogonial necrosis or by some combination of these. Depletion of the permanent type may be brought about in the same manner but with the added proviso that spermatogonial regeneration must also be prevented.

There is ample evidence on hand that exposure to x-rays brings about the onset of maturation depletion (see introduction for references). It then follows that the effects of a dose of x-rays large enough to produce a permanent absence of spermatogonia must differ quantitatively and/or qualitatively from the effects of a dose causing only temporary changes. The present work is concerned with

the response of the mouse testis to a dose of x-rays large enough to induce permanent depletion of the seminiferous tubules.

In order to facilitate comparison with the present results, a brief resumé of previous results utilizing a dose of x-rays of 320 r (Bryan and Gowen, 1956) is included here. With respect to interphasic spermatogonia, our 320 r data indicate a marked decline to less than 10% of the control value by three days following exposure. This low level remains in effect until 10 days, at which time regeneration commences. Spermatogonial mitotic activity follows a different course. There is an initial decline reaching a low point 8 hours after exposure, then a marked rise during the 8–24-hour period. This is followed by a further and more extensive decline during 1–3 days post-irradiation. Thereafter the pattern of response is essentially the same as for the non-dividing cells. The 2560 r data show marked deviations from this pattern. The area occupied by interphasic spermatogonia undergoes a rapid decline reaching a very low level by 5 days. There is a slight rise during the 5–10-day period, but the level then declines to zero by 16 days. The mitotic spermatogonia follow a similar pattern but the rate of decline during the early post-irradiation period (1–24 hours) is much more rapid. There is, then, no rise in mitotic activity during the 8–24-hour period; nor does repopulation of the seminiferous tubules take place. These facts lend themselves to the interpretation, that following exposure to 2560 r of x-rays, spermatogonial mitosis must be delayed or inhibited for a longer period of time than in the case of the 320 r experiment. Furthermore the surviving spermatogonia must be unable to sustain a regenerative phase after the initial inhibitory effects of the irradiation have worn off.

As stated above, spermatogonial necrosis may contribute to some extent to the depletion process. It is also to be expected that large doses of x-rays would be likely to produce a greater frequency of cell death than small doses. Hence it is probable that spermatogonial cell death may play a more prominent role in the initiation of maturation depletion following exposure to large doses of x-rays. With these points in mind, the pertinent sections were analyzed (by the Chalkley method) for spermatogonial and non-spermatogonial necrosis. These data are listed in Table II together with corresponding data obtained from the 320 r experiment. Exposure to either dose of x-rays increases the frequency of necrosis above control levels by one hour after irradiation. Similarly, a peak is reached at the 8-hour period followed by a return to lower levels at 24 hours. The pattern of response is therefore about the same for either dose of x-rays; however, there is a difference in the magnitude of this response. At 8 hours after irradiation with 2560 r, the frequency of spermatogonial necrosis is almost double that found following exposure to 320 r. Then at 24 hours following exposure no cells were encountered which could be classified as necrotic spermatogonia. These observations may be interpreted in two ways. On the one hand it may mean that a large fraction of cells are heavily damaged and undergo degenerative changes even though they are far removed, in time, from mitotic activity. The remaining cells, then, constitute a less severely damaged fraction which may undergo degeneration with the onset of mitosis. This interpretation is in accord with the views of Lasnitski (1943) concerning the effect of large doses of x-rays (2500–10,000 r). The possibility also exists that the observed absence of necrotic spermatogonia is correlated

with the very low mitotic rate 24 hours following exposure. This view receives some support from the reports of Glücksmann and Spear (1939) and others, to the effect that irradiation-induced cellular degeneration occurs at about the same time as the onset of mitotic activity. Then it follows that under conditions where mitotic levels are low (as in the present case) the chances of encountering necrotic cells are likewise much reduced. It is obvious from the present data that spermatogonial nuclei survive for different periods of time (some are present at 5–10 days following exposure). This must mean that some cells have suffered less damage than others, yet this fraction also is destined to be eliminated from the tubules by 16 days following x-ray exposure. From this we may conclude that severely damaged cells may undergo degeneration prior to the onset of mitosis (in agreement with Lasnitski), while less severely damaged cells do not degenerate until they attempt to enter mitosis.

With respect to the foregoing discussion, the relations between the levels of necrosis induced by different doses of x-rays are of significance. As Table II shows, an 8-fold increase in dose approximately doubles the frequency of necrotic cells at the 8-hour period. Although the conditions of the present experiments are quite different from those of Lasnitski (1943), who used tissue cultures of chick fibroblasts, nevertheless the results are fairly similar. This author's results indicate that a four-fold increase in dose increased the frequency of necrosis to about 1.4 times that of the low dose, whereas a 1.1-times increase in necrosis resulted when the dose was doubled. If a curve is fitted to these data it is found that an 8-fold increase in dose should result in the approximate doubling of the necrotic level (as observed in the present work). This offers further support for the idea that, as the x-ray dose is increased, cells further removed from the sensitive period are likely to suffer lethal injury.

It may be argued that fixed and stained preparations do not allow a very accurate estimation of the frequency of necrosis. Unless the intervals between the fixation times are less than the time necessary for cells to undergo lysis and be eliminated, such estimates are likely to be minimum values. However this is open to verification. Thus from control data the total area of the tubules occupied by spermatogonia (normal + necrotic) can be determined. A comparison of this value with similar determinations on irradiated material will reveal the goodness of fit existing between the expected and observed frequencies. The important point is whether or not any irradiation-induced decrease in area occupied by normal spermatogonia can be accounted for by an increase in the necrotic value. An analysis of this kind is summarized in Table III. It can be seen that, with the exception of the Ba 320 r, one-hour material, each time period shows a deficiency of spermatogonia. Following exposure to 320 r, these deficiencies range from about 10% to 20% of control values during the first 8 hours. With respect to the 2560 r experiment, the corresponding values range from about 12% to 26% of controls. It is clear that these changes in area are, during the first 8 hours, quite similar despite the difference in dose levels employed. Since the data in Table III take into account spermatogonial necrosis, the observed deficiencies cannot be accounted for on the basis of the increased frequency of necrosis as reported in Table II.

On *a priori* grounds it would be logical to impute the observed difference to

additional and "unobserved" spermatogonial necrosis. This is not an entirely satisfactory explanation. The stage at which cells are most sensitive to irradiation corresponds to that portion of the mitotic cycle during which chromosomal reduplication is taking place. Irradiation does not inhibit DNA synthesis in cells which are already in the period of synthesis, but delays these cells in entering division (see Howard and Pelc, 1953). At the time of irradiation, then, there is a fraction of spermatogonia which has passed the critical stage. These cells are most probably those observed in mitosis during the first few hours following exposure. In confirmation of this are the results of Bullough and Van Oordt (1950) and others, which indicate that, in the mouse, the duration of mitosis (prophase–telophase) is of the order of three hours. Now a certain proportion of these dividing spermatogonia transform into spermatocytes. These products of division are, therefore, lost from the spermatogonial fraction. Thus it follows that shortly after irradiation, the spermatogonial fraction will be decreased both by loss of these cells and by the reduction in frequency of replacement divisions. Unfortunately it is not possible to calculate the decrease in the spermatogonial fraction to be expected on these grounds. However it is evident that the deficiencies reported in Table III cannot entirely be ascribed to "unobserved" necrosis. The estimates of necrosis as determined in the present work are, therefore, reliable indices of irradiation-induced cellular degeneration.

The effects described above are cumulative, and therefore any deficiency should become progressively more marked with time following exposure to large doses of x-rays. Reference to Table III shows that this is the case. Exposure to 2560 r results in reduction of spermatogonial area to 20–27% of control values by 24 hours. In addition, it was expected that the 2560 r data would show trends similar to those following exposure to 320 r, but of greater magnitude. The data of Tables II and III are in agreement with this, but the relation is somewhat obscured by variation in the level of spermatogonia scored at 24 hours following exposure to 320 r. Since relatively few animals were used in these experiments, sampling errors undoubtedly contribute to this observed variation between strains.

In the case of the 320 r experiment, the surviving fraction of spermatogonia eventually repopulate the tubules. This suggests that recovery has occurred prior to the onset of mitotic activity 10 days following exposure. It is also possible to interpret these findings to mean that the surviving spermatogonia constitute a relatively more resistant fraction. Such an interpretation would be in accord with the conclusions of Eschenbrenner *et al.* (1948). In marked contrast are the results of the 2560 r experiment. Here, also, a small fraction of spermatogonia are present at 5 days following exposure. At 10 days, both strains show a slight increase in spermatogonia over the 5-day levels, but by 16 days, spermatogonia have been completely eliminated. These observations may be explained by the assumption of a further period of necrosis during the 10–16-day interval. This implies, in contrast to the 320 r case, that the spermatogonia present at 5 days subsequently attempt to undergo mitosis at which time latent damage expresses itself.

The spermatogonia present at 5 days following exposure to either dose of x-rays have the morphological characteristics of the so-called type A or "dusty" spermatogonia. Type A cells are regarded as "stem-line" germ cells by Clermont and Leblond (1953). These authors point out that a small fraction of Type A

spermatogonia after one division cycle become "dormant." Such "dormant" cells do not divide again until later in the spermatogenic cycle. In other words these spermatogonia remain "dormant" for about 6 days following the initiation of this inactive phase. This means that, in these cells, recovery from the effects of radiation exposure should be possible before mitosis recommences. With respect to our 320 r material, this apparently is the case. On the other hand, any recovery following exposure to 2560 r must only be partial since repopulation does not occur —despite the suggestion of an increase in frequency of spermatogonia by 10 days post-irradiation. On these grounds the spermatogonia present at 5 days following exposure to 2560 r (some or all of which may in fact be "dormant" type A cells) must be capable of limited division. Otherwise, the frequency of spermatogonia would not undergo the changes observed during the 5–16 day period.

Further evidence which points up the more widespread damage produced by exposure to this high dose of x-rays is provided by a consideration of non-spermatogonial necrosis. Reference to Table II shows that at 24 hours, the necrotic level has risen to 1.5–2.0 times that of the control. This increase can be largely accounted for on the basis of the very high frequency of degenerating metaphase I spermatocytes. It was observed that practically all metaphase I plates present in sections of these tubules were necrotic. In the 320 r experiment, the frequency of non-spermatogonial necrosis remained close to control levels. Very few necrotic metaphase I spermatocytes were encountered in this material.

The present data, when considered together with the results of our 320 r experiment, allow several conclusions to be drawn with respect to the manner in which the histological effects of radiation exposure are brought about. Certain of these conclusions take on added significance when considered in the light of other experimental approaches. Cells are prevented from entering division following irradiation. This may come about either through inhibition of DNA synthesis (chromosomal reduplication) or by death of the cells. Both mechanisms play a role in the irradiation-induced depletion of spermatogonia. The death of cells plays a more important role in this process following high doses of irradiation— such as 2560 r—than following exposure to low doses (320 r in the present case). Irradiation-induced mitotic inhibition would appear to be the major factor following exposure to relatively low doses of x-rays. As is readily apparent, this effect of x-rays on cells is of a very basic nature. It must perforce be taken into consideration if the nature of the effects of irradiation on biological systems is to be evaluated in a proper manner.

It is clearly evident that different levels of injury are produced by the irradiation treatments used here. Thus following 320 r, the surviving spermatogonial fraction is capable of mitotic activity to the extent necessary for the initiation of tubule repopulation. After exposure to 2560 r, on the other hand, the survivors are incapable of such a sustained effort.

Summary

1. Changes in the cellular composition of the seminiferous tubules induced by exposure to 2560 r of x-rays have been analyzed by a quantitative histological procedure. These data have been compared with the results obtained following ex-

posure to a much lower dose (320 r) in an attempt to gain further insight with respect to the manner in which the observed changes are brought about.

2. Exposure to 320 r results in a temporary maturation depletion of the seminiferous epithelium. This is brought about mainly by the inhibition of spermatogonial mitosis with irradiation-induced spermatogonial necrosis playing only a minor role. In contrast, exposure to 2560 r produces a permanent depletion due to the fact that surviving spermatogonia are incapable of sustained regenerative efforts.

3. The frequency of necrotic spermatogonia, following 2560 r, was found to be double the peak value attained in the 320 r material, or four times that of the corresponding controls.

4. Taken together, the data for the 320 r and 2560 r experiments suggest that spermatogonial depletion is brought about in two ways: (1) by suppression of mitosis due to inhibition of DNA synthesis, and (2) the killing of cells. Irradiation-induced necrosis plays a much more important role following exposure to high doses of x-rays. Even so the frequency of necrosis in either experiment did not reach very high levels, being about 9% after the low dose and about 15% in the case of the high x-ray dose.

5. Further evidence was obtained in support of the view that relatively heavily damaged cells may undergo degenerative changes prior to the onset of division, while less heavily damaged cells manifest degenerative changes only at about the time of entry into mitosis.

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